

# Induction of adenocarcinoma from hamster pancreatic islet cells treated with *N*-nitrosobis(2-oxopropyl)amine *in vitro*

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Our previous studies in the hamster pancreatic cancer model have indicated that pancreatic ductal adenocarcinomas derive not only from ductal/ductular cells but also from islets. To verify the presence of carcinogen-responsive cells within islets, we tested the effect of the pancreatic carcinogen *N*-nitrosobis(2-oxopropyl)amine (BOP) on recently established continuous hamster pancreatic islet culture. Isolated pure pancreatic islets of hamsters were treated *in vitro* with BOP at a concentration of 0.25 mM three times a week for 19 weeks. Each treatment week was designed as a stage. The growth of these cells, designated KL5B, was compared with untreated cultured islets, designated KL5N. As in our previous study, between 14 and 21 days of culture, exocrine and intermediary cells developed within both KL5N and KL5B islets, which were then replaced by undifferentiated cells. No differences were found in the growth patterns of KL5N and KL5B until stage 4, when KL5B cells showed accelerated cell growth and cell pleomorphism, which increased gradually at later stages of treatment. Anchorage-independent and *in vivo* growth did not appear until stage 19. Mutation of *c-Ki-ras* at codon 12 (GGT→GAT) was detected in KL5B cells but not in KL5N cells. *In vivo* KL5B cells formed anaplastic invasive cancer with areas of glandular formation, over-expressed TGF- $\alpha$  and EGFR, expressed cytokeratin, vimentin, laminin and  $\alpha$ -1 antitrypsin and reacted strongly with L-phytohemagglutinin and tomato lectin. Some cells within islets are responsive to the carcinogenic effects of BOP. Whether these cells represent islet cell precursors (stem cells) or malignant transdifferentiated islet cells remains to be seen.

## Introduction

The silent course of pancreatic cancer and its explosive fatal outcome have hampered our understanding of tumor histogenesis and early biochemical and genetic alterations,

**Abbreviations:** BOP, *N*-nitrosobis(2-oxopropyl)amine; SMG, submandibular gland; PHA, phytohemagglutinin.

information that could help us diagnose the disease at a curable stage. Although general opinion favors the hypothesis that pancreatic tumors originate from ductal/ductular cells in humans, the views are divided for experimental pancreatic cancer. Some believe in ductal genesis, whereas others suggest acinar cells are the precursor cells (1).

In the hamster model, which mimics human disease in many clinical and biological aspects, we have demonstrated that pancreatic adenocarcinoma originates not only from ducts and ductules (2–6), but also from islets (7–11). In fact, the first morphological change that occurs during pancreatic carcinogenesis is the appearance of ductular structures within islets (intra-insular ductules) or around the islets (peri-insular ductules) (2,4,6,8). The intra-insular ductules proliferate forming either benign patterns consistent with human pancreatic microcystic adenomas or becoming increasingly hyperplastic, dysplastic and atypical and culminating in the formation of malignant glands that destroy the islets and invade the surrounding tissues, even when they are of microscopic size (4–6,8). The following observations support the role of islets in pancreatic ductal carcinomas: (i) streptozotocin pretreatment, which causes destruction of  $\beta$ -cells, inhibits the pancreatic carcinogenicity of *N*-nitrosobis(2-oxopropyl)amine (BOP) at low doses or prevents it at high doses (12–14); (ii) genetically diabetic hamsters with atrophic islets are resistant to the pancreatic carcinogenic effects of BOP, whereas the pancreas of a non-diabetic strain with intact islets is susceptible (15); (iii) induction of nesidioblastosis enhances pancreatic carcinogenesis (16); and (iv) transplantation of homologous islets into the submandibular gland (SMG) of recipient hamsters and subsequent BOP treatment induces invasive and metastasizing ductal-type adenocarcinomas, histological and immunohistochemical analyses of which point to the derivation of tumors from within islets (9–11).

Our limited studies in humans have indicated that cancer cells also develop within islets (4,17). In a recent observation, both of the microscopical pancreatic carcinomas which we found incidentally at autopsy appeared to develop within islets (18). These observations led us to believe that certain pancreatic cells within islets are particularly sensitive to the carcinogenic insult.

To test this hypothesis, we established, for the first time, a continuous culture of hamster pancreatic islets (19). In this culture, the initially pure islets were replaced temporarily by ductal, acinar and intermediary cells (having the characteristics of both acinar and islet cells) before giving rise to undifferentiated cells, which seem to present as pancreatic stem cells. Although presently the origin of these cells from islet cell precursors or from transdifferentiation of islet cells is obscure, the model provided a unique opportunity to test the effect of BOP on cultured islets. Because it has been shown by us (20) and others (21) that BOP can transform isolated pancreatic ductal cells *in vitro*, we tested its effect on cultured hamster islets.

## Materials and methods

### Animals

Eight- to 10-week-old male Syrian golden hamsters from the Eppley colony were used for transplantation experiments. Animals were housed in cages with Sani-cell bedding and were kept under standard laboratory conditions (temperature  $20 \pm 2^\circ\text{C}$ , 10 air changes/min, 12 h/12 h light/dark cycle) and received pelleted diet (Wayne, Indianapolis, IN). Water was provided *ad libitum*.

### Chemicals

BOP was synthesized in our laboratories. The culture medium (M3:6) was a gift from InCell (San Antonio, TX). Penicillin, streptomycin, trypsin, EDTA, fetal bovine serum and RPMI 1640 were purchased from Life Technologies (Gaithersburg, MD). Purified agar used for soft agar assay was ordered from Difco (Detroit, MI). PCR core reagents were purchased from Perkin-Elmer Cetus (Norwalk, CT).

### Islet culture

Two hundred freshly isolated islets, designated KL5N (no BOP treatment), and 250 islets designated KL5B (with BOP treatment), were cultured in M3:6 medium supplemented with 6% fetal bovine serum, 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin. The islets were placed in a polystyrene Petri dish (Baxter, McGaw Park, IL) on a rocker (15 r.p.m./min) at  $37^\circ\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$  in air for 14 days, as reported (19). In none of the islets that were examined immunohistochemically and electron microscopically after isolation was any exocrine cell contamination noticeable. There were no cells reactive to pancytokeratin, a marker for hamster pancreatic ductal/ductular cells or that presented features of ductal or acinar cells. The floating islets were hand picked every day to separate them from the attached fibroblasts and were placed in a new dish with fresh M3:6 medium. On day 14 after the initial isolation, when no fibroblasts could be identified, the islets were plated into a six-well tissue culture plate, allowed to attach and continuously cultured without shaking. Thereafter, they were trypsinized and subcultured in M3:6 medium every week.

### BOP treatment

Islets were treated with 0.25 mM BOP from the first day of islet culture, designated KL5B cells. A pilot study had shown that this concentration of BOP has no toxic effects and is well tolerated by islet cells. The BOP-containing medium was changed for fresh every day for the first 14 days, the period during which fibroblasts were eliminated. Thereafter, the medium was changed three times per week (Mondays, Wednesdays and Fridays) for fresh BOP. This treatment scheme was designated a stage, which was longitudinally continued in subsequent culture, as reported (20). After the end of each stage, the cells were transferred (passaged) into a new flask. For better comparison of data at each stage, the KL5N cells were also transferred (passaged) to a new flask whenever the KL5B cells were passaged. Consequently, the stage of KL5B cells corresponded to the passage of KL5N cells. Like KL5N cells, when islets were transferred to a flask, they attached to the bottom of the flask and at day 21 (stage three) a mass of cells radiated from islets into the surrounding culture. The cell aggregate representing the remnant of islets disappeared and at stage 4 the culture consisted of a monolayer of undifferentiated cells. These cells were trypsinized and counted. A portion of the cells (between  $3 \times 10^3$  and  $3 \times 10^4$  cells) were harvested for soft agar assay, for examination of the c-Ki-ras mutation and, if the cell number was sufficient, for immunohistochemistry and electron microscopy analysis. After stage (passage) 19, BOP treatment was discontinued and further cell transfer was defined as passage.

### Anchorage-independent growth assay

This assay was performed as reported earlier (20). Colony formation was checked once a week and discarded after 45 days.

### Electron microscopical examination

Transmission electron microscopical examination was performed according to our published technique (3,22).

### Cell growth determination

The growth curve of the cells in culture was established by plating 50 000 and 200 000 cells into separate T-25 plastic flasks. The number of viable cells, determined by the dye exclusion test, was counted at 24 h intervals using a hemocytometer. Population doubling time was calculated by plotting the mean cell number versus days in culture on a semi-logarithmic graph, as reported (21,22).

### Transplantation experiment

Approximately 1 000 000 KL5B cells at stage 19 were transplanted into the subcutaneous tissue, pancreas or SMGs of recipient hamsters (three hamsters per site), as reported (23). Subcutaneous masses and lesions in the SMG and

pancreas were removed at autopsy and all organs were examined grossly for metastases. The regional lymph nodes of these hamsters and the pancreas of three age-matched control hamsters were removed, fixed in buffered formalin and processed for histology by conventional methods.

### Histochemical and immunocytochemical examination

All histochemical and immunocytochemical examinations, including the multilabeling technique, were performed as previously described (24). Antibodies used in the experiment are reported in our earlier study (19). The immunoreactivity was scored as none (–), weak (+), moderate (++) or strong (+++). The cellular immunoreactive site was defined as cytoplasmic, luminal or cell membrane. To calculate the number of stained KL5N and KL5B cells, two observers counted 1000 cells on each slide and the number of cells immunoreactive with a given antibody was estimated as a percentage. The average value was considered representative.

### Cytogenetic analysis

The karyotyping of KL5N and KL5B cells at passages 24 and 34 was performed, as reported (20,22). Fifty-one KL5B and 42 KL5N cells were examined.

### DNA and RNA purification

DNA and total RNA from the pancreas, islet and KL5N cells were purified by minor modifications in the guanidine isothiocyanate/acid phenol method (27) published previously (28). Briefly, tissue or cells were homogenized in a buffer containing 4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% sarcosyl and 0.1 M 2-mercaptoethanol. It is acidified by adding 2 M sodium acetate (pH 4.0) and then phenol/chloroform extracted and ethanol precipitated.

### Examination of the c-Ki-ras mutation

Mutation of the c-Ki-ras gene was examined by RT-PCR, according to a previously described method (20).

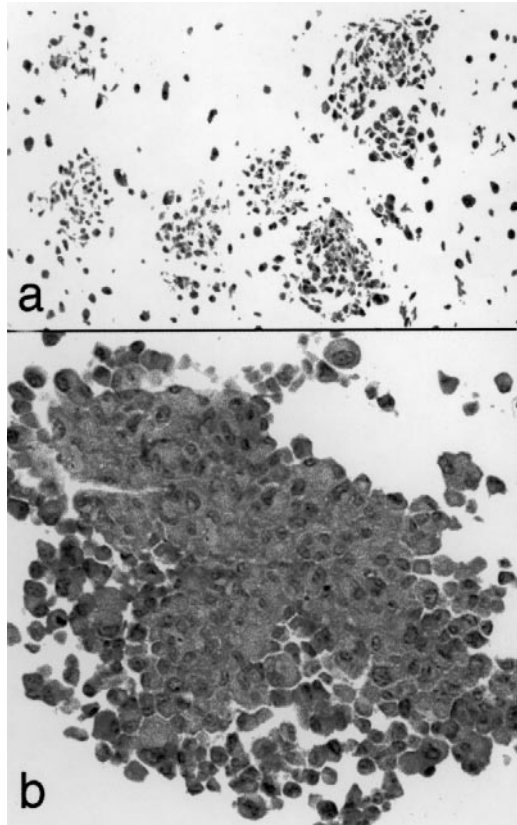
### RT-PCR

One microgram of total RNA was combined with 100 ng random hexamer primers and the solution was heated at  $72^\circ\text{C}$  for 5 min and then placed on ice. dNTPs (10 mM each), reverse transcriptase, RNasin,  $10\times$  PCR buffer, 25 mM  $\text{MgCl}_2$  and water were added (as per the manufacturer's instructions; Perkin-Elmer, Norwalk, CT) and the mixture was incubated at  $42^\circ\text{C}$  for 60 min. The cDNA synthesis reaction was terminated by heating at  $95^\circ\text{C}$  and the mixture was stored at  $-70^\circ\text{C}$ . PCR was performed using 10  $\mu\text{l}$  cDNA in a total reaction volume of 50  $\mu\text{l}$  containing 2.5 U Taq DNA polymerase, Taq buffer containing 1.5 mM  $\text{Mg}^{2+}$ , 0.6  $\mu\text{M}$  forward primer, 0.6  $\mu\text{M}$  reverse primer and 200  $\mu\text{M}$  dNTPs. A hot start technique was used. Thirty-five cycles of amplification were performed of  $95^\circ\text{C}$  for 1 min,  $55^\circ\text{C}$  for 90 s and  $72^\circ\text{C}$  for 290 s, with a final elongation for 10 min. A negative control lacking template was used in the reaction. Products were analyzed by electrophoresis on 1.5% agarose gels. Primers for PCR of IPF1, IEF1, NKx6.1, Neuro-D, Pax6, EGFR and TGF- $\alpha$  are shown below. These primer sequences were obtained from hamster (NKx6.1), rat (IPF1, IEF1, EGFR and TGF- $\alpha$ ) and mouse (NeuroD and Pax6). The primer sequences are: IPF1, forward 5'-CTCGCTGGGAACGCTGGAACA-3', reverse 5'-GCTTTGGTGGATTTCATCCACGG-3'; IEF1, forward 5'-ACCCTTACCAATGACTCCTATG-3', reverse 5'-ATGATGACTGCAAAATCGC-3'; NKx6.1, forward 5'-TCTTCTGCGCCGGGTGATG-3', reverse 5'-AGCCGCGTGCTTCTCTCTCCA-3'; NeuroD, forward 5'-CTTGCCCAAGAACTACATCTGG-3', reverse 5'-GGA-GTAGGGATGCACCGGAA-3'; Pax6, forward 5'-TTGGGAAATCCGAGACAGAT-3', reverse 5'-GGTACTGGAAGTCCCGGGGACTA-3'; EGFR, forward 5'-ACTGGCCTTAGGGAATGCC-3', reverse 5'-TCCTGTACACCCGCGGCACA-3'; TGF- $\alpha$ , forward 5'-ACCTGCAGGTTTTTGGTGCAG-3', reverse 5'-GGAGGGCGCTGGGCTTCTCG-3'.

## Results

### Islet culture

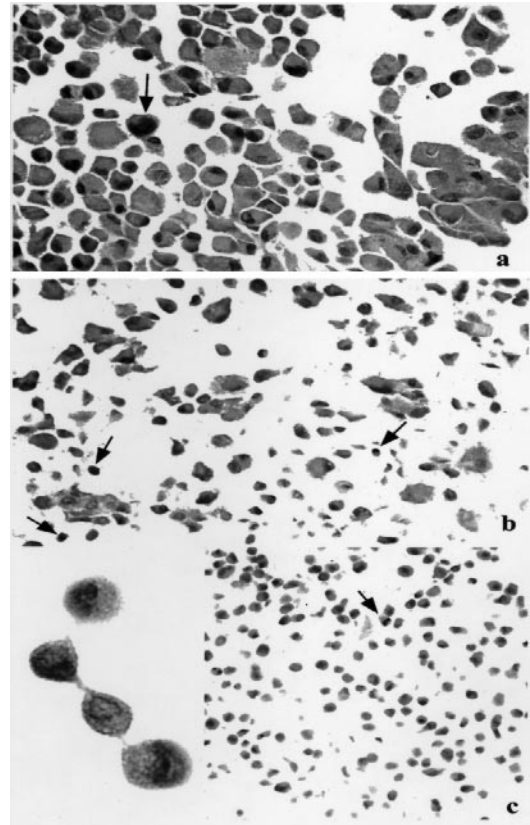
The growth patterns of both KL5N and KL5B islets were similar to those observed in our previous study (19). The size of islets varied between 0.12 and 0.32 mm and there were no differences in the size of islets between BOP-treated and untreated. At day 14 (stage 2), the number of floating islets transferred to flasks was 90 and 180 for the KL5N and KL5B cultures, respectively. As in our previous report (19), ductular, acinar and intermediary cells appeared within both KL5N and KL5B islets between stages 2 and 3. In both cultures, the epithelial cells from islet cores spread out into the surrounding



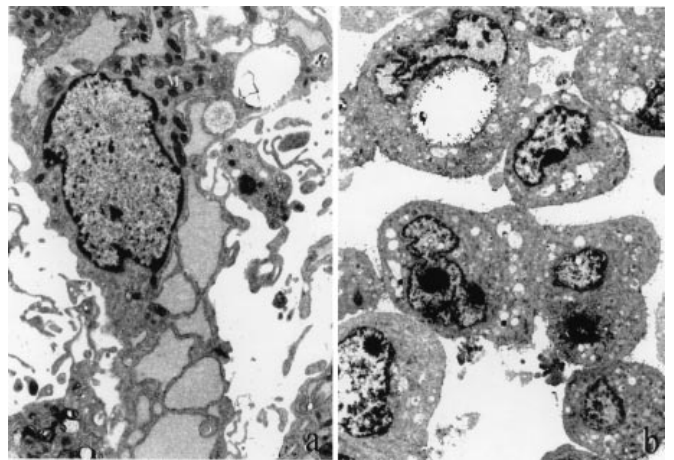
**Fig. 1.** KL5N cells (islets without BOP treatment) in culture. (a) At passage 4, the cells that spread out of islets attach to each other to make island-like aggregates (H&E,  $\times 40$ ). (b) At later passages, they form uniform cells with a tendency to adhere to each other (H&E,  $\times 210$ ).

area and attached to the bottom of the flask. At stage 4, although KL5N cells tended to attach to each other and grew in islet-like cell aggregates (Figure 1), KL5B cells formed a monolayer of pleomorphic cells with abundant eosinophilic cytoplasm and a few pleomorphic and hyperchromatic nuclei (Figure 2a).

At stage 8, the growth of KL5B cells accelerated, showing a doubling time of 36 h, compared with 48 h for KL5N cells. Although KL5N cells retained their monomorphic phenotype, KL5B cells presented as a small cell population with hyperchromatic nuclei between large cells (Figure 2b). The number of small cells increased gradually and, at stage 19, the culture was composed entirely of small cells with pleomorphic and hyperchromatic nuclei and scanty eosinophilic cytoplasm. Fine cytoplasmic attachments could be seen between some of these cells (Figure 2c). Whereas the growth and phenotype of KL5N cells at passage 19 remained fairly constant, that of KL5B cells further accelerated (doubling time 26 h). Also in contrast to KL5N cells, KL5B cells showed, for the first time, anchorage-independent growth and could be maintained in RPMI 1640 culture medium supplemented with fetal calf serum. Electron microscopically, the patterns of KL5N cells were similar to the cultured islet cells in our previous study (19) and presented as undifferentiated cells poor in cell organelles. KL5B cells, however, were pleomorphic and contained large vesicles, small cystic spaces containing material of various optical densities, microfilaments and a few intracytoplasmic lumens (Figure 3). No endocrine granules were detected.



**Fig. 2.** KL5B cells (islets treated with BOP) in culture. (a) At stage 4, the cells around the islets have large eosinophilic cytoplasm and hyperchromatic nuclei of various sizes. In a few cells, the nuclei occupied a larger portion of the cell (arrow). No *c-Ki-ras* mutation was found in these cells. (b) At stage 8, smaller cells appeared between the large cells. In some, the small cytoplasm was occupied by a large and hyperchromatic nucleus (arrows). No signs of malignancy and mutation were found. (c) At stage 19, small cells dominated the culture. The nuclei were hyperchromatic and pleomorphic and a few showed mitotic figures (arrow). At high power view (inset), the cells showed fine cytoplasmic processes between the cells. All sections were stained with H&E; (a–c)  $\times 210$ ; (c inset)  $\times 400$ .



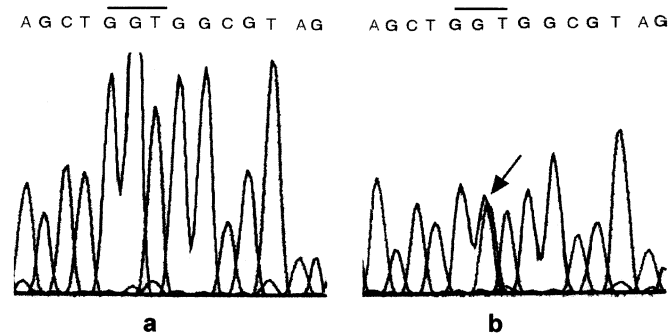
**Fig. 3.** Electron microscopical appearance of KL5B cells. (a) The small cells at stage 8 had a narrow cytoplasmic rim containing many mitochondria (M) and cystic structures filled with material of medium electron density.  $\times 7100$ . (b) At stage 19, the malignant cells were pleomorphic with irregular nuclei, cytoplasmic vesicles and a few intracytoplasmic lumens (upper left).  $\times 3000$ .

#### Activation of the c-Ki-ras oncogene

Mutation of c-Ki-ras was found only in KL5B cells at stage 19 (Figure 4). The mutation was in codon 12 (GGT→GAT), as found in primary pancreatic cancers, in cell lines derived from them (25) and in BOP-induced tumors arising from islets *in vivo* (10).

#### Immunohistochemical findings

In contrast to normal pancreatic islets, cultured islets did not react with anti-pancytokeratin. With KL5N cells after stage 3, only a few cells in islets but none in the monolayer showed reactivity with antibodies against insulin, glucagon or somatostatin. We did not use anti-chromogranin, because the antibody does not recognize hamster cells. Because of the availability of cells, further immunohistochemical examination was performed only in cells of stage 4 and thereafter. The immunoreactivity of KL5N cells to the antibodies was similar to that published earlier (19). No differences were found in the staining of KL5N and KL5B cells, except that more KL5B cells expressed laminin and TGF- $\alpha$  than KL5N (Table I). KL5B cells were reactive with antibodies against laminin, vimentin and  $\alpha$ 1-antitrypsin. In the normal pancreas, ductal, ductular, centroacinar and islet cells stained strongly with anti-pancytokeratin (Figure 5), whereas cytokeratins 14 and 18



**Fig. 4.** An electropherogram showing nucleotide sequences around codon 12 (GGT) of the c-Ki-ras gene (wild-type) in KL5N (a) and KL5B (b) cells. Two peaks (G and A) were seen in mutant-type (KL5B) cells at base 19. The codon GGT is mutated to the codon GAT.

were present only in ductal cells (Table II). The cells also showed strong binding to tomato lectin and phytohemagglutinin (PHA) (Figure 6).

#### *In vivo growth patterns of KL5B cells*

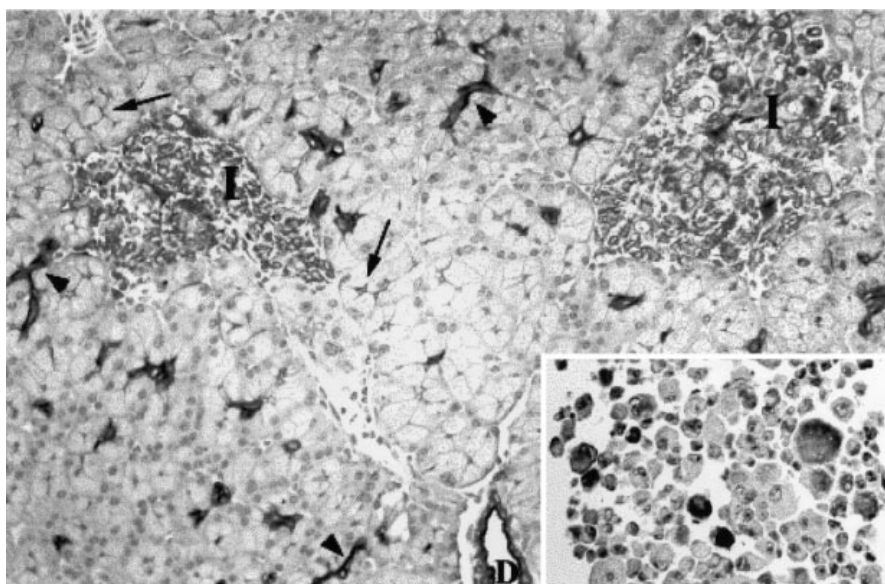
Subcutaneous tumors grew to a 20 mm mass within 2 weeks (Figure 7). At this time, all nine hamsters with KL5B cell transplants were autopsied. In all of these hamsters, invasive tumors were found, depending on the inoculation site, in the subcutaneous tissue, pancreas or SMGs. The subcutaneous tumors were encapsulated but had invaded the abdominal muscles (Figure 7). Tumors in the pancreas and SMGs had invaded the surrounding tissues, but no metastases were detected. Histologically, tumors of all three sites were anaplastic with a focal area of glandular formation (Figure 8). Profound vascularization, hemorrhage and necrosis were found within all tumors. Immunohistochemical findings in tumors of different transplantation sites are summarized in Table I. Except for the antibodies listed in Table I, no reactivity was seen with the remaining antibodies. A different degree of reactivity of the tumors was seen with EGFR, tomato lectin and PHA.

#### Cytogenetic analysis

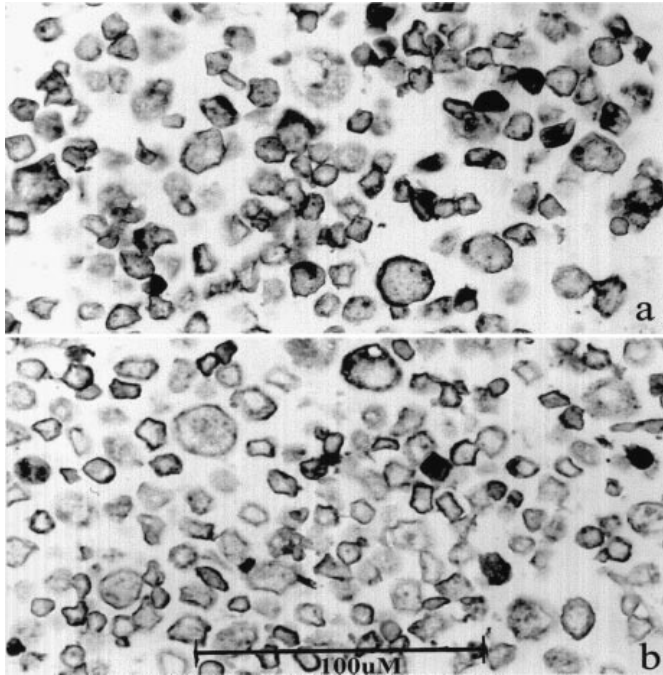
Cytogenetic analysis of KL5N at passages 27 and 34 showed the same pattern of the 45, XY normal hamster chromosome complement (22,26), with no detectable numerical or structural anomaly. Forty-five KL5B cells of passage 27 represented an abnormal clone characterized by a missing Y, monosomy 7 and 11, one copy of two markers and two copies of another marker (Figure 9). Six cells were a tetraploid version of this clone. The same patterns were found in KL5B cells of passage 34.

#### Expression of islet-specific genes

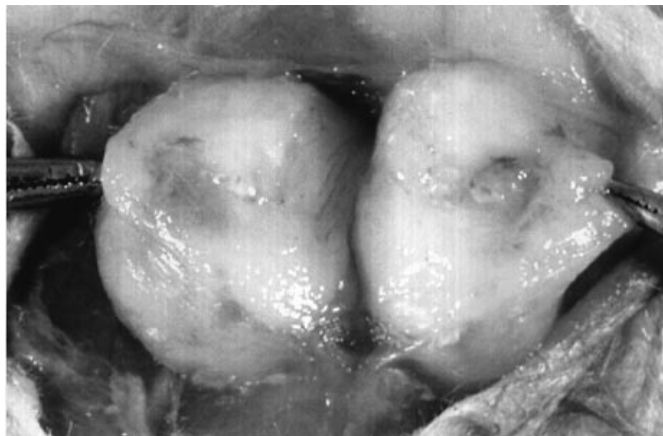
We analyzed the expression of insulin, IPF1, IEF1, NeuroD, Pax6 and NKx6.1 in normal pancreas, freshly cultured islets and undifferentiated islet culture KL5N and KL5B cells by RT-PCR analysis, as shown in Table III. Expression of insulin, IPF1, NeuroD, Pax6 and Nkx6.1 was seen in the normal pancreas and islets. However, these genes were not expressed in KL5B cells. Interestingly, the IEF1 gene was expressed in



**Fig. 5.** Immunoreactivity of the normal pancreatic tissue with antibody against pancytokeratin. Note the strong staining of a duct (D), ductules (arrowheads), centroacinar cells (arrows) and islet cells (I). At stage 4 and later ~20% of KL5B cells reacted with this antibody (inset). ABC method,  $\times 120$ ; inset,  $\times 210$ .



**Fig. 6.** Reactivity of KL5B cells at passage 32 with PHA (a) and tomato lectin (b). The reactivity of both lectins was mostly with the cell membrane although in a few cells it was diffuse cytoplasmic.



**Fig. 7.** Vertical section of a subcutaneous tumor in a hamster that was inoculated with KL5B cells at stage 19. The 2 cm tumor was encapsulated, firm and showed areas of hemorrhage.

the normal pancreas, islets and KL5B cells. Tubulin expression was seen in all the samples under study. EGFR and TGF $\alpha$  were expressed in pancreas, islets and undifferentiated cells.

## Discussion

Several studies in our laboratories have focused on the role of the islets in pancreatic carcinogenesis in the hamster model. In particular, promotion of pancreatic carcinogenesis by stimulating islet cell proliferation (16) and by inducing ductal-type cancers in the SMGs of hamsters bearing homologous islet transplantation (9–11) strongly supported this view. However, the question as to whether the tumors arose from some components attached to the transplanted islets (ductular or acinar cells) could not be ruled out with certainty. However, based on our observation of >500 isolated islets, the possibility of exocrine cell contamination cannot exceed one islet in 100.

**Table I.** Immunohistochemical reactivity of antibodies to normal hamster pancreas, KL5N and KL5B cells (passage 32)

| Antibody               | Normal pancreas<br>(% cells stained) | KL5N<br>(% cells stained)     | KL5B<br>(% cells stained)  |
|------------------------|--------------------------------------|-------------------------------|----------------------------|
| Pancytokeratin         | +++ <sup>a,b</sup>                   | +++ <sup>a</sup> (30)         | +++ <sup>a</sup> (20)      |
| Cytokeratin 14         | +++ <sup>a,*</sup>                   | +++ <sup>a</sup> (20)         | +++ <sup>a</sup> (10)      |
| Cytokeratin 18         | +++ <sup>a</sup>                     | +++ <sup>a</sup> (5)          | +++ <sup>a</sup> (10)      |
| Carbonic anhydrase II  | + <sup>**</sup>                      | + (20)                        | + <sup>a</sup> (20)        |
| Laminin                | +++ <sup>a,b</sup> (100)             | +++ <sup>a</sup> (50)         | +++ <sup>a</sup> (100)     |
| Vimentin               | +++ <sup>c</sup> (100)               | +++ <sup>a</sup> (70), + (20) | +++ <sup>a</sup> (100)     |
| Tomato lectin          | +++ <sup>c</sup> (100)               | +++ <sup>a,d</sup> (100)      | +++ <sup>a,d,f</sup> (100) |
| PHA                    | +++ <sup>f</sup> (100)               | +++ <sup>d</sup> (100)        | +++ <sup>d</sup> (100)     |
| TGF- $\alpha$          | + <sup>g</sup> (20)                  | +++ <sup>a</sup> (30)         | +++ <sup>a</sup> (70)      |
| EGFR                   | –                                    | + <sup>d</sup> (60)           | +++ <sup>a</sup> (80)      |
| $\alpha$ 1-Antitrypsin | + <sup>b</sup> (100)                 | + <sup>a,d</sup> (90)         | +++ <sup>a,d</sup> (100)   |

–, no staining; +, weak staining; ++, moderate staining; +++, strong staining.

\*Staining of ductal, ductular, centroacinar and islet cells

\*\*Staining of ductal cell only.

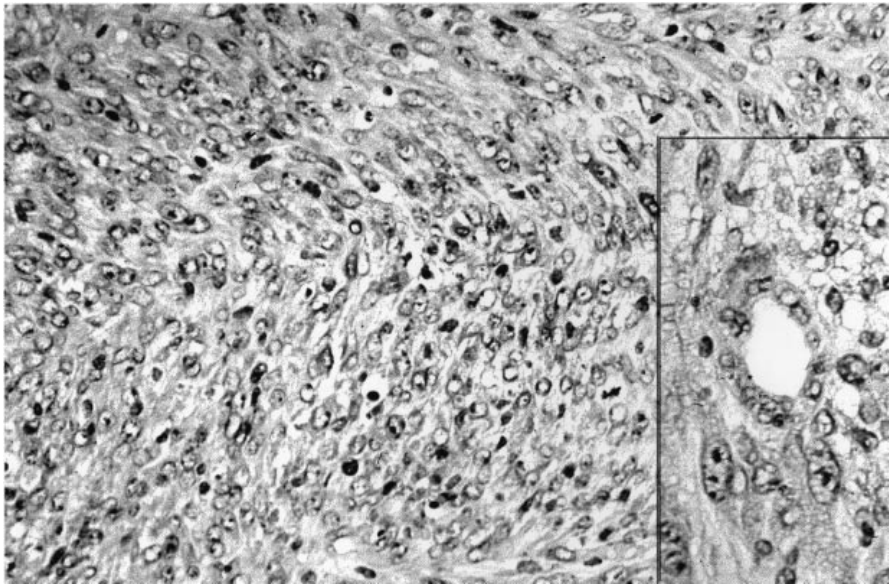
<sup>a</sup>Diffuse cytoplasmic; <sup>b</sup>acinar, islet and ductal cells staining; <sup>c</sup>staining of smooth muscles only; <sup>d</sup>cell membrane staining; <sup>e</sup>diffuse cytoplasmic staining of zymogen granules and of islet cells, luminal staining of ductal cells; <sup>f</sup>diffuse cytoplasmic staining of zymogen granules and islet cells; <sup>g</sup>staining of glucagon cells only.

This negligible level of impurity cannot explain the massive cell migration from all islets that are attached to the bottom of the flask into the surrounding culture (19). Moreover, according to our experience the growth of cells deriving from islets is significantly different from that of cultured ductal cells, in that ductal cells form glandular structures even in monolayer (27).

Because it has been shown that BOP can transform hamster ductal cells *in vitro* (20,21), it was of interest to examine the effect of BOP on isolated pure islets *in vitro* in the system we have established (19). In this system, isolation and long-term maintenance of pure islets free of exocrine cell contamination was possible.

Confirming our previous study (19), the present experiment demonstrates that islet cells in culture give rise temporarily to exocrine cells but culminate in the formation of undifferentiated cells, possibly representing stem cells. Although the dose of BOP used is relatively high compared with doses that reach the pancreas after *in vivo* treatment, no sign of toxicity was seen when compared with the KL5N cells. This could be the result of reduced levels of BOP-metabolizing enzymes in cultured cells, a possibility supported by the requirement for larger doses of BOP for *in vitro* transformation of ductal cells (20,21), while *in vivo* a single treatment with BOP is sufficient to induce tumors. There were no differences in the initial growth of islets with or without treatment with BOP, although it has been shown that carcinogens initially inhibit cell division by interacting with cellular DNA (28). There were also no differences in morphological and subcellular levels between BOP-treated and untreated cells until stage 4, when pleomorphic epithelial cells appeared in the KL5B cultures. This phenotypical change and the loss of carbonic anhydrase II was obviously unrelated to the c-Ki-ras mutation, which was first detected much later, at stage 19. It is likely that other as yet unknown genetic abnormalities preceded the c-Ki-ras mutation. Hence, it appears that differences exist between the malignancy of human and hamster pancreatic cells, because in humans mutation of c-Ki-ras occurs early and even in histologically





**Fig. 8.** The patterns of the subcutaneous tumor in Figure 7. Histologically, the tumor was anaplastic with large pleomorphic cells and many mitotic figures (~20 mitotic cells in 25× objective). Large necrotic and hemorrhaging areas were present in the center and glandular formation at the edge of the tumor (inset). H&E, ×210.

**Table II.** Comparison of the immunohistochemical reactivity of antibodies to KL5B cells (passage 32) injected s.c. into the submandibular gland or pancreas and grown *in vitro*

| Antibody           | Subcutaneous<br>(% cells stained) | Submandibular gland<br>(% cells stained) | Pancreas<br>(% cells stained) | <i>In vitro</i><br>(% cells stained) |
|--------------------|-----------------------------------|--|-------------------------------|--------------------------------------|
| Pancytokeratin     | + <sup>a</sup> (10)               | + to +++ <sup>a</sup> (10)               | + to +++ <sup>a</sup> (20)    | +++ <sup>a</sup> (20)                |
| Cytokeratin 13     | + <sup>a</sup> (5)                | + <sup>a</sup> (5)                       | + <sup>a</sup> (2)            | –                                    |
| Cytokeratin 18     | + <sup>a</sup> (10)               | + <sup>a</sup> (10)                      | + <sup>a</sup> (10)           | +++ <sup>a</sup> (10)                |
| Carbonic anhydrase | –                                 | + <sup>a</sup> (4)                       | + <sup>a</sup> (5)            | + <sup>a</sup> (20)                  |
| Laminin            | + <sup>a</sup> (100)              | + <sup>a</sup> (100)                     | + to +++ <sup>a,b</sup> (100) | +++ <sup>a</sup> (100)               |
| Vimentin           | + to +++ <sup>c</sup> (100)       | +++ <sup>c</sup> (100)                   | + to +++ <sup>c</sup> (80)    | +++ <sup>a</sup> (100)               |
| Tomato lectin      | ++ to +++ <sup>d</sup> (100)      | + to +++ <sup>b</sup> (100)              | + to +++ <sup>d</sup> (100)   | +++ <sup>a</sup> (100)               |
| PHA                | + <sup>e</sup> (100)              | +++ <sup>c</sup> (100)                   | +++ <sup>c</sup> (100)        | +++ <sup>a</sup> (100)               |
| EGFR               | + <sup>a</sup> (30)               | + <sup>a</sup> (60)                      | + <sup>a</sup> (80)           | + <sup>a</sup> (80)                  |
| α1-Antitrypsin     | + <sup>a</sup> (70)               | +++ <sup>a</sup> (80)                    | + <sup>a</sup> (60)           | +++ <sup>a</sup> (100)               |

–, no staining; +, weak staining; ++, moderate staining; +++, strong staining.  
<sup>a</sup>Diffuse cytoplasmic; <sup>b</sup>weak staining of tumor cells, strong staining of blood vessels; <sup>c</sup>granular cytoplasmic staining; <sup>d</sup>cell membrane and vascular staining; <sup>e</sup>cell membrane staining.



**Fig. 9.** The chromosomal pattern of KL5B at stage 27 showing 45, X, -Y, -7, -11, +mar1, + mar2×2, + mar3.

**Table III.** Expression of insulin and insulin-associated transcriptional factors in normal hamster pancreas, islets and KL5B cells derived from islets

| Factor        | Pancreas | Islets | KL5N | KL5B |
|---------------|----------|--------|------|------|
| IPF1          | +        | +      | –    | –    |
| IEF1          | +        | +      | +    | +    |
| Insulin       | +        | +      | –    | –    |
| Neuro-D       | +        | +      | –    | –    |
| Pax6          | +        | +      | –    | –    |
| NKx6.1        | +        | +      | –    | –    |
| EGFR          | +        | +      | +    | +    |
| TGF- $\alpha$ | +        | +      | +    | +    |

normal-appearing cells (29,30). Perhaps, certain environmental factors lacking under the *in vitro* conditions are necessary for this mutation.

The study nevertheless confirms that pancreatic islet cells can give rise to ductal-type cancer cells. However, we do not yet know whether the tumor progenitor cells derive from stem cells within islets or from transdifferentiated islet cells. Pancreatic exocrine cells as a contaminant of islets can be ruled out because our immunohistochemical examination using pancytokeratin, a marker for hamster pancreatic ductal cells, and electron microscopic examination of isolated islets and those examined 7 days later in culture did not show any evidence of cell contamination. All the exocrine and intermediary cells developed within islets. The origin of the exocrine cells from intra-insular ductules can also be ruled out, because intra-insular ductules develop only in aged hamsters and under some pathological conditions. In the present study, however, we used islets of healthy and young hamsters, which do not contain intra-insular ductules. Moreover, in our hands BOP-transformed hamster ductal cells lack the *c-Ki-ras* mutation (20). Consequently, the cells that develop from cultured islets appear to be different from ductal/ductular cells.

What was remarkable was the *in vivo* growth pattern of KL5B cells. They formed anaplastic invasive tumors, similar to those induced by BOP in islets growing in the SMG of hamsters (10,11) and to PC-1.0 cells, derived from a primary BOP-induced pancreatic cancer (31). The anaplastic and invasive nature of the KL5B tumor could be related to massive overproduction of TGF- $\alpha$ , which was shown to be overexpressed in metastasizing ILA cells (32) derived from tumors induced in the SMG of hamsters after homologous islet transplantation (9–11) and in human pancreatic cancer cells (33–35). The remarkable vascularization of the tumors could also be related to TGF- $\alpha$  (35). The expression of  $\alpha$ 1-antitrypsin in both KL5N and KL5B cells but not in any adult hamster pancreatic cells further supports the origin of these cells from a primitive precursor cell. This acute phase reactant protein has been found in human pancreatic tumors assumed to derive from stem cells, including solid cystic (papillary) tumor (36) and pancreatoblastoma (37). Hence, this protein, as well as vimentin, not present in pancreatic parenchymal cells, also appears to present as a marker for hamster pancreatic stem cells (38). Interestingly,  $\alpha$ -fetoprotein, another protein produced in fetal tissues, has recently been found in human pancreatic cancer (39), an indication that human pancreatic cancer cells also derive from primitive pancreatic cells.

Simultaneous expression of vimentin and cytokeratin, also found in aggressive human breast cancer (40), seems to be

associated with increased invasive behavior. The same seems to apply to the lack of blood group A antigen expression. We have shown that well-differentiated, BOP-induced tumors and the slow growing PC-1.0 cell line derived from a primary BOP-induced cancer (41) consistently express blood group A antigen (42), whereas poorly differentiated, fast growing PC-1.0 cells derived from an s.c. transplant of a primary hamster pancreatic cancer (31) do not. However, in contrast to PC-1.0 cells, which express blood group A antigen expression when transplanted into hamsters (23), KL5B cells failed to produce this antigen *in vivo*. Hence, it appears that in KL5B cells the genes involved in blood group A synthesis are inactivated. Whether these genes were located in the missing or altered chromosomes is unclear. Nevertheless, like ILA cells, KL5B cells were also missing a Y chromosome, a finding that was not seen in transformed ductal cells (20). Interestingly, the missing sex chromosome has been found to be one of the most frequent findings in human pancreatic cancer (43–45). However, because deletion of a Y chromosome can occur in other types of cancer, particularly often in metastases of colon cancer (45), this abnormality is not specific for pancreatic cancer and its role in the evolution of pancreatic cancer is unknown. In human pancreatic cancer, enhanced expression of EGFR has been found to correlate with alterations of chromosome 7 (46), one of which was missing in KL5B cells. Because expression of EGFR appeared early in both KL5B and KL5N cells, a correlation between this chromosomal damage and EGFR expression is unlikely.

There were some significant differences in the chromosomal changes between TAKA-1-BOP tumors arising from hamster pancreatic ductal cells (22), from tumors in SMGs of hamsters bearing transplanted islets (32) and in KL5B cells. These include deletion of one of the chromosomes 4 and 7 only in KL5B cells, alteration of chromosome 3 only in ILA and TAKA-1-BOP cells and many extra chromosomes in ILA cells (32). However, because of a limited number of cells examined from each cell line, it is unclear whether these abnormalities are homogenous or merely heterogeneous.

The malignant alteration of islets by BOP in culture unequivocally points to the ability of islet cells to metabolize BOP. Consequently, it appears that both hamster ductal cells, which can also be transformed by BOP in culture (20), and islets have the necessary metabolizing enzyme and, thus, are direct targets of BOP.

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